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Uptake of 3,5,3′-L-Triiodothyronine into Bullfrog Red Blood Cells Mediated by Plasma Membrane Binding Sites

Kiyoshi Yamauchi, Ryuya Horiuchi[1], Sakuji Koya and Hiroo Takikawa

Institute of Endocrinology, Gunma University, Maebashi 371, Japan

ABSTRACT—To examine the mechanism of thyroid hormone transport into the bullfrog red blood cells (RBCs), the uptake of 3,5,3′-L-triiodothyronine (T_3) into the intact RBCs of bullfrog and the binding of T_3 to the purified plasma membranes were studied. The specific T_3 uptake into the adult RBCs was saturable at a certain T_3 concentration and temperature-dependent showing maximum binding at 15°C. The uptake of iodothyronines into RBCs showed stereospecificity for T_3 ($T_3 \gg$ 3,5,3′-D-triiodothyronine = L-thyroxine). The nuclear uptake of T_3 was only a few percent of the cellular associated T_3 after incubating at 15°C for 1 hr. Monodansylcadaverine, an inhibitor of receptor-mediated endocytosis, blocked T_3 uptake into the adult RBCs even at 0°C, with a half maximal inhibitory concentration of 65 μM. The effect of MDC strongly suggests that even at 0°C T_3 should enter bullfrog RBCs by receptor-mediated endocytosis. To clarify the presence of the T_3 binding sites on the plasma membranes, we purified the plasma membranes from adult RBCs. Scatchard analysis revealed the presence of two classes of T_3 binding sites on the plasma membrane: a high affinity-low capacity site (Kd_1: 36.0 ± 12.7 nM, MBC_1: 14.8 ± 6.7 fmol/μg protein), and a low affinity-high capacity site (Kd_2: 5.34 ± 1.54 μM, MBC_2: 2.44 ± 0.51 pmol/μg). From these results it is suggested that T_3 initially binds to the specific binding sites on the cell surface of the bullfrog RBCs, and then enters the cells by receptor-mediated endocytosis.

INTRODUCTION

It has been indicated that the nuclei of tadpole red blood cells (RBCs) contain putative thyroid hormone receptors [1], and the number of the receptors increases corresponding to the enhancement of the 3,5,3′-L-triiodothyronine (T_3) concentration in plasma during metamorphosis and decreases after metamorphosis [2, 3]. These results suggest that T_3 might regulate the metabolism of the differentiated RBCs.

It is known that the plasma membranes from human [4, 5], rabbit [6] and rat RBCs [7] contain specific binding sites for T_3. Further, in mammalian cells, many evidences have been accumulated that T_3 binding to the membrane associated proteins on the cell surface should be the initial step in its actions on the target cells, and the uptake of T_3 into the cells might be mediated by endocytosis [8–12].

In the case of bullfrog RBCs, it was reported that T_3, but not L-thyroxine (T_4), was transported into RBCs by a carrier mediated pathway that is not dependent on metabolic energy [13]. However, details of the thyroid hormone transport pathways into bullfrog RBCs are still unclear. In order to clarify this problem, the mechanism of T_3 uptake into the intact RBCs closely related to the plasma membranes was examined.

MATERIALS AND METHODS

Reagents [^{125}I]T_3 (3380μCi/μg) was purchased from New England Nuclear (Boston, U.S.A.). T_3, 3,5,3′-D-triiodothyronine (D-T_3), T_4 and Monodansylcadaverine (MDC) were purch-

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[1] To whom reprint requests should be addressed.

ased from Sigma (St. Louis, U.S.A.). Dibutyl phthalate and Dinonyl phthalate were obtained from Eastman Kodak (Rochester, U.S.A.). MS-222 (tricaine methane sulfonate) was purchased from Sankyo (Tokyo, Japan).

Preparation of RBC *Rana catesbeiana* were collected from ponds in Misato, Saitama Pref., Japan. The adult frogs were anesthetized by immersing in 0.2% MS-222, and the heart areas were exposed. Blood samples were obtained by cardiac puncture with heparinezed syringes. The RBCs were separated from plasma by centrifugation at 1,500×*g* for 15 min at 4°C, followed by washing three times with TNC buffer (15 mM Tris-HCl, 108 mM NaCl, 0.77 mM $CaCl_2$; pH 7.4). The number of RBCs was determined with a Coulter Counter (Coulter Electronics, U.S.A.).

Preparation of the plasma membranes of RBC The plasma membrane fractions of RBCs were obtained by osmotic lysis and discontinous sucrose density gradient centrifugations according to the method of Okazaki *et al.* [14] with slight modifications. In brief, the washed RBCs were poured into 20 volumes of stirring ice-cold Solution A (10 mM Tris-HCl, 20 mM $MgCl_2$ and 5 mM dithiothreitol, pH 7.4). The nucleated RBC ghosts were then pelleted by centrifugation at 15,000×*g* for 20 min. After washing the ghosts several times with Solution A, the precipitate was resuspended in 5 volumes of Solution B (Solution A containing 50 mM sodium bisulfite) and sonicated. The resultant suspension was layered over a step gradient of 20% (w/w) and 60% sucrose, and then centrifuged at 80,000×*g* for 90 min. The membrane fraction at the interface of the sucrose solutions was further layered over a step gradient of 20%, 30%, 40%, 50% and 60% sucrose. After centrifugation at 80,000×*g* for 2 hr, the membrane fraction at the 40–50% interface was immediately used for $[^{125}I]T_3$ binding expriments. Protein content was determined by the method of Bradford [15] using bovine γ-globulin as the standard protein.

Uptake of $[^{125}I]T_3$ by intact cells RBCs (2.5×10^5 cells) were incubated in 250 μl of TNC buffer containing 0.1% glucose and 0.1 nM $[^{125}I]T_3$ in the presence or absence of 20 μM unlabeled T_3 at 15°C. Cellular bound $[^{125}I]T_3$ and free $[^{125}I]T_3$ were separated by the oil-centrifugation method according to Horiuchi *et al.* [10] with slight modifications. In brief, after incubation of RBCs with $[^{125}I]T_3$, 200 μl of the chilled cell suspension was applied on the top of a three-layer solution in an Eppendorf 400 μl micro test tube. The three-layer solutions from top to bottom consisted of 40 μl of dibutyl phthalate : dinonyl phthalate (6.5:3.5, v/v); 100 μl of the buffer containing 0.25 M sucrose, 0.5 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.4); and 30 μl of dibutyl phthalate : dinonyl phthalate (8: 2, v/v), respectively. The cells and free $[^{125}I]T_3$ were immediately separated by centrifugation at 14,000 ×*g* for 2 min at 4°C. The tips of the tubes containing the cell pellet were cut off. The cellular $[^{125}I]T_3$ values were determined in a Beckman Gamma 8000 spectrometer. Non-specific $[^{125}I]T_3$ association with the cells calculated from the samples in the presence of 20 μM unlabeled T_3 was less than 5% of total $[^{125}I]T_3$ uptake.

Binding of $[^{125}I]T_3$ to nuclei of RBCs After incubation of RBCs with 0.2 nM $[^{125}I]T_3$ at 15°C for various periods, the cells were washed twice with TNC buffer, and the pellets were treated twice with 2 ml of the buffer containing 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 15 mM 2-mercaptoethanol, and 0.5% Triton X-100 [16] at 0°C. The isolated nuclei were collected by centrifugation at 400×*g* for 5 min and $[^{125}I]T_3$ bound to the nuclei was determined.

Binding of $[^{125}I]T_3$ to plasma membranes The purified membranes (10 μg protein in 250 μl of TNC buffer) from bullfrog RBCs were incubated with 0.2 nM $[^{125}I]T_3$ in the presence of various concentrations of unlabeled T_3 at 15°C. After incubation, 50 μl of 5 mg/ml bovine γ-globulin and 300 μl of cold 12% polyethylene glycol 6000 in TNC buffer containing 0.2 M $ZnCl_2$ were added to the chilled incubation mixture. The resultant suspension was centrifuged at 1,500×*g* for 10 min at 4°C. The pellet was washed once with 2 ml of 6% polyethylene glycol containing 0.1 M $ZnCl_2$, and bound $[^{125}I]T_3$ in the pellet and free $[^{125}I]T_3$ were detemined. Non-specific binding calculated from the samples in the presence of 20 μM unlabeled T_3 was 40–50% of total binding, which was

subtracted from total binding. The values for the dissociation constant (Kd) and maximum binding capacity (MBC) were calculated from Scatchard plot by computer analysis according to the method of Rosenthal [17].

RESULTS

The characteristics of T_3 uptake into adult RBCs $[^{125}I]T_3$ uptake into adult RBCs at 15°C increased with time and reached a plateau after 15 min as shown in Figure 1A. The specific T_3 uptake was approximately 95% of the total uptake. As shown in Figure 2, the T_3 uptake was temperature-dependent showing maximum at 15°C, but it decreased to be 68% of the maximum uptake at 30°C. The $[^{125}I]T_3$ uptake into the nuclei was slow and only 2–3% of the total cellular $[^{125}I]T_3$ incubated at 15°C for 2 hr, and then the uptake gradually increased to 12% of the cellular T_3 after 8 hr (Fig. 1B).

Figure 3 shows the competitive inhibition of $[^{125}I]T_3$ uptake into RBCs by the iodothyronines. The inhibition was stereospecific for T_3 and fifty percent of the specific cellular uptake of $[^{125}I]T_3$ was inhibited by 28 nM unlabeled T_3, 3.0 μM T_4 or 3.6 μM D-T_3. These results indicate that T_3 is stereospecifically transported into the bullfrog RBCs.

To examine the possibility of the receptor-mediated uptake of T_3 into bullfrog RBCs, the effect of MDC, an inhibitor of receptor-mediated endocytosis [9], on $[^{125}I]T_3$ uptake into RBCs was examined. As shown in Figure 4, the specific $[^{125}I]T_3$ uptake at 0°C was almost completely inhibited by MDC, and 50% of the specific $[^{125}I]T_3$ uptake was inhibited by 65 μM MDC. When incubated at 15°C for 30 min, similar results were obtained (data not shown).

The characteristics of T_3 binding to the plasma membranes of RBCs Figure 1C shows that $[^{125}I]T_3$ binding to the purified plasma membranes at 15°C increased with time and reached a plateau after 30 min. $[^{125}I]T_3$ binding to the plasma membranes was specifically inhibited by unlabeled T_3. From the concentrations of unlabeled iodothyronines required to depress 50% of the specific

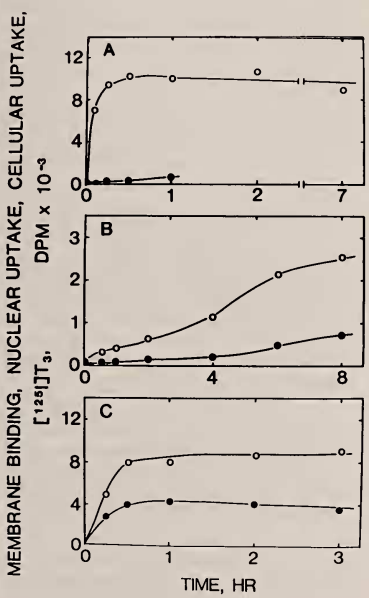

FIG. 1. Time course of $[^{125}I]T_3$ uptake into the bullfrog RBCs and its binding to the plasma membranes. (A) $[^{125}I]T_3$ uptake into the intact RBCs. Bullfrog RBCs (2.5×10^5 cells) were incubated with 0.1 nM $[^{125}I]T_3$ at 15°C for the indicated periods. The cellular uptake of $[^{125}I]T_3$ was determined by the oil-centrifugation method as described in "Meterials and Method". (B) $[^{125}I]T_3$ uptake into nuclei. Cells (2.5×10^5 cells) were incubated with 0.2 nM $[^{125}I]T_3$ at 15°C for the indicated periods. The nuclei were isolated and the radioactivity incorporated into the nuclei was determined as described in "Materials and Methods". (C) $[^{125}I]T_3$ binding to the plasma membranes. The purified plasma membranes (10 μg protein) from bullfrog RBCs were incubated with 0.2 nM $[^{125}I]T_3$ at 15°C. After washing the membranes with 6% polythelene glycol, 0.1 M $ZnCl_2$ in TNC buffer, the bound $[^{125}I]T_3$ was determined as desctibed in "Materials and Methods". (●——●): in the presence of 20 μM unlabeled T_3. (○——○): in the absence of unlabeled T_3. Each point in the figures represents the mean for triplicate tubes with a standard error less than 5%.

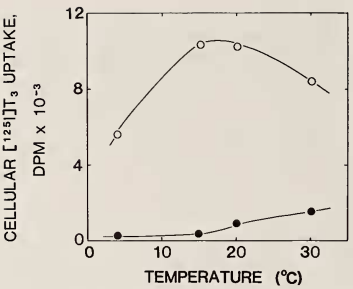

FIG. 2. Temperature-dependent uptake of $[^{125}I]T_3$ into the bullfrog RBCs. The bullfrog RBCs (2.5×10^5 cells) were incubated with 0.1 nM $[^{125}I]T_3$ for 30 min at 4, 15, 20 or 30°C with (●) or without (○) 20 μM of unlabeled T_3. The cellular $[^{125}I]T_3$ uptake was determined as desctibed in "Materials and Methods". Each point represents the mean for triplicate tubes with a standard error less than 5%.

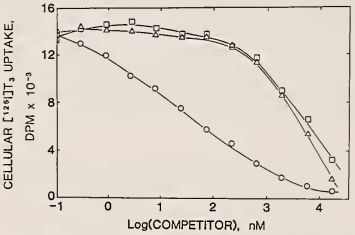

FIG. 3. Competitive inhibition of $[^{125}I]T_3$ uptake into bullfrog RBCs by various iodothyronine analogues. The bullfrog RBCs (2.5×10^5 cells) were incubated with 250 μl of 0.1 nM $[^{125}I]T_3$ for 30 min at 15°C in the presence of various concentrations of L-T_3 (○), T_4 (△) or D-T_3 (□), respectively. $[^{125}I]T_3$ uptake was determined as desctibed in "Materials and Methods". Non-specific association was calculated to be 5% of total uptake from the samples with 20 μM unlabeled T_3 and was subtracted from total $[^{125}I]T_3$ uptake. Each point represents the mean for triplicate determinations with a standard error less than 5%.

binding of $[^{125}I]T_3$, it is roughly estimated that the affinities of D-T_3 and T_4 were 1/7 and 1/70 of T_3, respectively (data not shown). A typical Scatchard plot of T_3 binding to RBC membranes at 15°C is

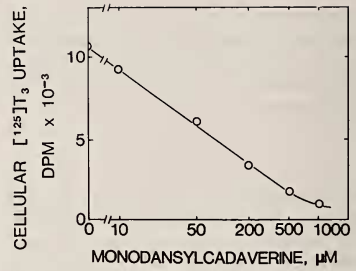

FIG. 4. Inhibition of $[^{125}I]T_3$ uptake into bullfrog RBCs by MDC. The bullfrog RBCs (2.5×10^5 cells) were incubated with 0.1 nM $[^{125}I]T_3$ in the presence of various concentrations of MDC for 3 hr at 0°C. Cellular associated $[^{125}I]T_3$ was determined by the oil-centrifugation method as described in "Meterials and Methods". Each point is the mean for triplicate determinations with a standard error less than 10%. Non-specific association determined from the samples incubated with 20 μM T_3 was subtracted.

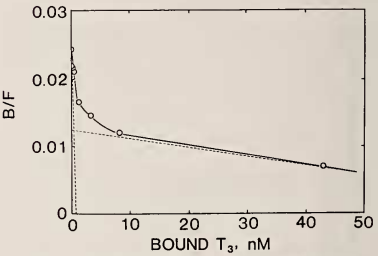

FIG. 5. Scatchard plot of T_3 binding to the plasma membranes of bullfrog RBCs. The membranes (10 μg protein) prepared from bullfrog RBCs were incubated with 0.2 nM $[^{125}I]T_3$ at 15°C for 2 hr in the presence of various concentrations of unlabeled T_3. $[^{125}I]T_3$ binding to the membranes were determined by the method of centrifugation as described in "Materials and Methods". Each point represents the mean for triplicate determinations. The solid line is the theoretical curve analyzed by computer for two classes of binding sites according to Rothental [17]. The broken lines are the Scatchard plot resolved into two lines showing the high affinity-low capacity site and the low affinity-high capacity site.

shown in Figure 5, revealing the presence of two classes of binding sites. The mean values for

binding of [^{125}I]T_3, it is roughly estimated that the affinities of D-T_3 and T_4 were 1/7 and 1/70 of T_3, respectively (data not shown). A typical Scatchard plot of T_3 binding to RBC membranes at 15°C is shown in Figure 5, revealing the presence of two classes of binding sites. The mean values for dissociation constants (Kds) and maximum binding capacities (MBCs) from three separated experiments were 36.0±12.7 nM (Kd_1) and 14.8±6.7 fmol/μg protein (MBC_1) for a high affinity-low capacity site, and 5.34±1.54 μM (Kd_2) and 2.44±0.51 pmol/μg protein (MBC_2) for a low affinity-high capacity site, respectively.

DISCUSSION

Recently much evidence has been accumulated showing that T_3 specific binding sites are on the plasma membranes of many mammalian cells. T_3 binding to the binding sites on the cell surface has been considered to be the initial step in T_3 actions on the target cells. However, in poikilothermal animals, the pathway of thyroid hormone from plasma into the cells is still unclear. The putative thyroid hormone receptors in the nuclei of hepatocytes from pre- and pro-metamorphic tadpoles have been characterized *in vitro* [18]. However, the prepared hepatocytes were proved to be heterogeneous. Further, the hepatocytes from the tadpole would be easily damaged by extensive perfusion. To clarify the thyroid hormone transport system on the cell surface, bullfrog RBCs could be a good model because the nuclear thyroid hormone receptors have been characterized in the RBCs of metamorphic tadpoles and adult frog [1–3], and the pure intact RBCs could be easily obtained by simple centrifugations. Furthermore, it was demonstrated by indirect immunofluorescent staining of blood smear that T_4 distributed not only in the plasma but within the RBCs, where it appeared at the edge of the nucleus and around the plasma membrane [19]. In this study, we demonstrated the presence of T_3 specific binding sites on the plasma membrane of bullfrog RBCs and further showed that receptor-mediated endocytosis might be the major pathway of the thyroid hormone uptake into bullfrog RBCs.

The uptake of T_3 into the intact bullfrog RBCs was saturable, temperature-dependent and stereospecific, as shown in Figures 2 and 3. T_3 uptake into the cells was maximal at 15°C and the cellular uptake reached a plateau after 30 min at this temperature. Non-specific association was less than 5% of total cellular T_3, and 15% of the [^{125}I]T_3 added to the incubation medium was accumulated to RBCs under the experimental conditions (Fig. 1A). Considering that the separation of cellular associated T_3 and free T_3 in the incubation medium by the oil-centrifugation method is very rapid to be done within 30 sec, the dissociation of cellular [^{125}I]T_3 might be negligible during the experimental process. These data indicate that the T_3 uptake into RBCs is by active transport but not by simple diffusion.

However, the distribution of T_3 to the nuclei was small and slow: only 2–3% of cellular T_3 was distributed to the nuclei after 2 hr and the nuclear distribution was increased to 12% after 8 hr at 15°C. In the case of mammalian cells, the distribution of T_3 to the nuclei of GH_3 cells was reported to reach a plateau at 10% of the cellular T_3 within 1 hr [10]. These data suggest that the capacity of nuclear T_3 receptor is only a part of the whole cellular binding capacity of mammalian and also amphibian cells. The binding to the purified plasma membranes and the uptake into the intact cells are both stereospecific for T_3. The order of the stereospecificity of the uptake into the cells and the binding to the purified plasma membrane are similar (T_3>D-T_3≧L-T_4). These results might evidence that T_3 enters the cells after having bound to the T_3 specific binding sites on the plasma membrane.

In bullfrog tadpole RBCs, Galton *et al.* [13] reported the presence of a single, T_3 specific but energy-independent T_3 uptake pathway with a Km of 50 nM from the kinetic analysis at 22°C. However, they did not attach importance to the binding sites on the cell surface. From the equilibrium binding of T_3 to the plasma membranes purified from RBCs, we showed the presence of two classes of binding sites: a high affinity-low capacity site (Kd_1: 36 nM, MBC_1: 14.8 fmol/μg protein) and a low affinity-high capacity site (Kd_2: 5.34 μM, MBC_2: 2.44 pmol/μg protein). Although a comparison of the Kd value of T_3 binding to the

plasma membrane with the Km value of T_3 uptake is not warranted because they were obtained at the different conditions, the Km value corresponded well with the Kd_1 value in our study. Thus, the uptake of T_3 into bullfrog RBCs might occur via the high affinity-low capacity site on the plasma membrane. Considering that the affinity of the low affinity site is far weaker than those of thyroid hormone binding proteins in plasma [20], the contribution of the low affinity site on the T_3 uptake pathway of bullfrog RBCs could be little.

The binding of iodothyronines to the purified plasma membrane from bullfrog RBCs was stereospecific for T_3 and the affinity of L-T_4 was 1/70 of T_3. In mammalian RBCs, the presence of two classes of T_3 binding sites on the plasma membranes from human [4] and rat RBCs [5] was reported. We also found that the stereospecificity and affinity of T_3 binding to bullfrog RBC membranes were similar to those of T_3 binding to the human RBC ghosts [21].

We showed a further link between the binding sites for T_3 on the plasma membranes and the uptake of T_3 into the intact RBCs. MDC is known to be an inhibitor of the internalization of α_2-macroglobulin, epidermal growth factor or vesicular stomatitis virus into mouse fibroblasts [9]. MDC was also shown to inhibit internalization of T_3 bound to the plasma membrane into GH_3 cells, a rat pituitary tumor cell line [10], and mouse fibroblasts [11] with a half-maximal concentration of 29 and 90 μM, respectively. However, MDC showed no effect on rat GH_3 cells treated at 0°C. In the case of bullfrog RBCs, as shown in Figure 4, MDC almost completely inhibited the cellular association of T_3 at 0°C with a half-maximal concentration of 65 μM. A similar effect was seen when incubated at 15°C (data not shown). From these results, it was strongly suggested that the T_3 uptake into bullfrog RBCs was performed by receptor-mediated endocytosis even at 0°C. The difference between the feature in bullfrog and mammlian cells could partially depend on the components and fluidity of the plasma membranes. These results might support the probability that T_3 would be effective even at 0°C and this might be physiologically significant in poikilothermal animals including frogs.

In mammalian RBCs, we know the T_3 actions which are not mediated by nuclear receptors, such as the stimulation of glucose uptake [22], Ca^{2+}-ATPase activity [23], or the enhancement of oxygen consumption [24]. In the case of bullforg RBCs, it was shown that the life span of the RBCs of tadpole form was shorten and the production of the RBCs of adult form was induced by *in vivo* treatment with T_4 [25]. Moreover, Thomas *et al.* [26] showed that T_3 induced the synthesis of T_3 receptors in RBC nuclei. We showed in this study the evidences indicating that T_3 is actively transported from plasma into RBCs, and strongly suggested the possibility of the receptor-mediated endocytosis of T_3 into RBCs. However, direct relevance of the T_3 actions of bullfrog RBCs and T_3 transport pathways, especially T_3 binding to the plasma membrane, is remained to be clarified in future.

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